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Creation of a functioning chimeric mammalian kidney

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Creation of a functioning chimeric mammalian kidney. The possibility of adding new nephrons to the mammalian kidney was studied. Embryonic metanephric tissue was implanted into the renal cortex of neonatal mice less than 24 hours old, and the development of the chimeric kidney was followed over the following two to four weeks. Donor tissue was obtained from the homozygous beige mouse and a mouse line transgenic for the β-globin gene, which provided distinct cellular and nuclear markers which were used to distinguish donor from recipient nephrons. Differentiation and growth of donor nephrons occurred in the host kidney and included vascularized glomeruli, mature proximal tubules, and tubular extensions into the renal medulla. Glomerular filtration was demonstrable in donor nephrons using FITC-dextran as a marker of filtration into the proximal tubules. Transplantation of metanephric tissue into adult mouse kidneys did not lead to glomerular or tubular differentiation. This study demonstrates the feasibility of adding functioning nephrons to mammalian kidneys in species in which there is ongoing nephrogenesis post-natally.

The possibility that organ function can be modified by the introduction of cells has recently been raised by the demonstration that retrovirally infected cells overexpressing specific genes can be integrated into the bone marrow [1], brain [2], skin [3] and blood vessels [4]. The complexity of the renal architecture, however, limits the feasibility of random integration of cells into the kidney if the goal is to augment nephron function or to exploit the ability of genetically-modified renal cells to deliver molecules into the urine or into the peritubular circulation. To circumvent this problem, a theoretically viable approach would be to integrate entire functioning nephron units into the kidney.

The present study examined the feasibility of adding new nephrons to the mammalian kidney. The end-points which were aimed for were anatomical differentiation of the nephron and evidence of glomerular filtration. Chimeric kidneys were created by implantation of embryonic metanephric tissue into the newborn mouse kidney. In this species nephrogenesis continues into the postnatal period. Nephrons of donor origin integrated into the vascular system of the host kidney and underwent anatomical differentiation. Glomerular filtration occurred in some of these nephrons.

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Methods

Experimental strategy

Metanephric tissue from donor embryonic mice, 13 to 16 days postcoital (pc), was implanted into the renal cortex of neonatal mice within 24 hours of birth to create a chimeric kidney. The donor cells possessed a distinct marker which allowed the donor origin of the tissue to be established when the chimeric kidneys were harvested two to four weeks later.

Donor and recipient mice

Transgenic mouse line 83 as donor. The transgenic mouse line 83 was created by and provided by Cecilia Lo (University of Pennsylvania, Philadelphia, USA). This line was created by electrophoretic injection of mouse β-globin DNA into the male pronucleus of hybrid SJL × C57BL/6 mouse zygotes. The cells of the resulting founder generation contain approximately 1000 tandemly-repeated copies of the plasmid inserted at the telomere of chromosome 3. In this model, the DNA is not expressed but can be used as a cell marker by means of in situ hybridization with a mouse β-globin genomic DNA probe [5, 6] (Fig. 1a). Fertility is low in the homozygotes and in order to generate sufficient donor tissue, homozygous male or female mice were crossed with CBA mice of the opposite sex. The hemizygous metanephric tissue of the F_1 offspring of these matings was transplanted into outbred Q strain mice. (Pilot studies revealed complete absence of cellular rejection within the first three weeks of transplantation so that there was no requirement for histocompatibility in these short-term studies.)

Homozygous beige mouse line as donor. The C57BL6 bg^J/bg^J beige mouse, an animal model of the Chediak-Higashi syndrome was obtained from the Jackson Laboratories, Maine, USA. Affected mice have giant lysosomes which are prominent in pigment cells, in liver parenchymal cells, and in the cytoplasm of renal proximal tubular cells particularly in the pars recta. The abnormal lysosomes can be identified as giant granules by enzyme histochemistry, electron microscopy and autofluorescence (Fig. 1b), and are thus suitable markers for chimeric studies [7–9]. Although the kidneys of these mice become pigmented with age [10], no renal pathology has been reported. Beige mouse metanephric tissue was transplanted into congenic neonatal C57BL/6J mice.

Control operations. These were performed on the recipient strains of the same age and included the entire surgical procedure but without the insertion of the metanephric tissue.

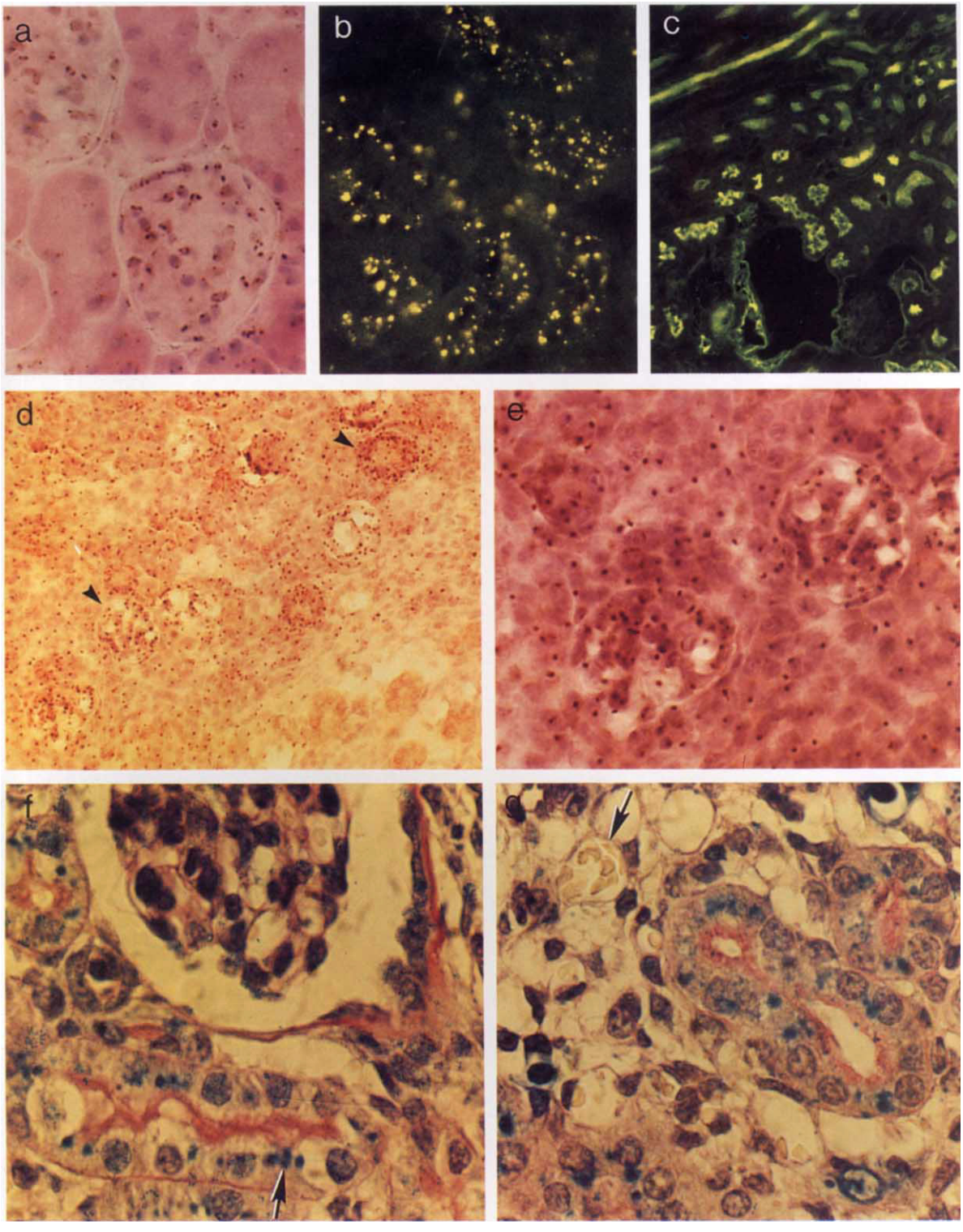

Fig. 1. a. *Adult kidney from transgenic mouse line 83.* In situ hybridization signal for β-globin insert shows as a brown dot over each nucleus. Note that this mouse was homozygous and thus has two signals per nucleus. (× 320) **b.** Adult beige mouse kidney. Proximal tubules contain giant yellow autofluorescent granules. (× 320) **c.** Adult C57BL/6J mouse kidney after FITC dextran injection. Note green fluorescence in lumens of proximal tubules and lack of autofluorescent granules in cytoplasm. (× 128) **d.** Chimeric mouse kidney. Upper left of panel shows transgenic donor tissue positive for the β-globin insert, and includes glomeruli with open capillaries (lower arrow) and non-vascularized glomeruli (upper arrow). Recipient tubules which lack the transgenic signal are seen at lower right. (× 128) **e.** Chimeric kidney. Donor glomeruli with open vascular loops derived from transgenic mouse metanephros identified by a positive in situ hybridization signal. (× 320) **f.** Chimeric kidney. Shows glomerulus with opening to proximal tubule in upper half of panel. Below is a beige donor proximal tubule with blue galactosidase positive lysosomes within cytoplasm (arrow). (PAS × 800) **g.** Chimeric kidney. A mature beige phenotype proximal tubule with brush border surrounded by loose mesenchyme-like tissue containing vascular channels (arrow). (PAS × 800)

Harvesting of embryonic kidneys

Mice were time-mated, with day 0 being the day of the vaginal plug. At days 13 to 16 pc the pregnant animals were sacrificed by cervical dislocation, the embryos removed and the two kidneys dissected from their surrounding tissues and transferred to a dish containing DME/Hams F12 medium (1:1) supplemented with 10 mM HEPES buffer and sodium bicarbonate (1.1 mg/ml) [11]. Each metanephros was cut symmetrically into four or eight segments (depending upon the age of the embryo) each of which included both cortex and medulla. The segments were kept in this medium at 4°C for up to two hours, which was the average operating time for a litter of 5 to 10 mice.

Operative procedure

Neonatal mice less than 24 hours old were anaesthetized by cooling in a glass dish mounted on ice. When there was no spontaneous movement and no response to a pin prick the left kidney was exposed by a flank incision and a segment of donor metanephros inserted into a tunnel fashioned in the cortex of the host organ. After suture of the abdominal musculature and the skin, the wound was painted with New Skin (Beecham) and the animal returned to its mother. More than 95% of animals survived the surgical procedure but only approximately 50% survived the first 24 hours. Thereafter, the mice grew normally.

A small number metanephric transplants were performed in adult, mice (25 g) anaesthetized with phenobarbital sodium (0.5 mg/10 g body wt) using the same surgical procedure.

Nine neonatal recipients of transgenic tissue, 15 neonatal recipients of beige tissue, 8 adult recipients and 20 control mice survived for two to four weeks after which time the operated kidney was removed and processed for histological examination.

Demonstration of glomerular ultrafiltration

Mice that were four weeks of age were large enough to undergo this procedure without difficulty. Four-week-old transplant recipients were anaesthetized with intraperitoneal phenobarbital sodium and the inferior vena cava exposed by a mid-line abdominal incision. FITC-dextran (molecular wt 10,000) (Molecular Probes Inc.) was injected as 1 mg intravenous bolus in 0.1 ml 0.9% saline. After 15 seconds the chimeric kidney was removed and processed as described below. In paraffin sections the FITC-dextran appears within the lumen of the proximal tubule as a bright green fluorescence when viewed under ultraviolet excitation (Fig. 1c). (If nephrectomy is performed at 5 to 10 seconds after injection the fluorescence is located with in the glomerular vasculature. This timing agrees with a previous study which directly observed the passage of intravenously injected dextrans through the nephron of the rat [12]). Since this technique allows both the autofluorescent beige granules and the fluorescent dextran within the tubular lumen to be viewed in the same section, evidence for glomerular filtration was only studied in beige transplants.

Because it was theoretically possible that uncomplexed fluorescein might enter the lumen by tubular secretion [13], the FITC-dextran was injected into three mice, 12 hours after ureteral ligation of the right kidney. The fluorescence appeared in the tubule lumen of the intact kidney, but in no instance did it appear within tubule lumens of the right, non-filtering kidney, where it was confined to the glomerular and peritubular capillaries. The appearance of green fluorescence within the lumen of the proximal tubule was thus considered to be unequivocal evidence of the ultrafiltration of dextran into the tubule via the glomerulus.

Histology

Routine fixation and paraffin embedding was performed on formalin-fixed kidneys unless otherwise stated. Sections were cut serially at 5 μ through the entire chimeric kidney and were stained with hematoxylin and eosin or PAS. Unstained sections were used for detection of autofluorescent beige granules, the low background fluorescence being adequate to outline the anatomical structure of the tubules. Photomicrographs were taken with Kodak Ectachrome P1600 for epifluorescence microscopy and Kodachrome ASA 64 for light microscopy.

Detection of donor-cell markers

Demonstration of β-globin DNA in transgenic cells by in situ hybridization. The kidney was fixed in 3:1 ethanol:glacial acetic acid and embedded in paraffin wax (Fig. 1a). Sections were transferred to poly-L-lysine-coated glass slides, dried, dewaxed, washed in 3% H_2O_2 in methanol and then rinsed in ethanol and dried. The DNA was denatured in 70% formamide in 2 × SSC at 70°C for five minutes, then quenched in cold 70% ethanol and dehydrated through an ethanol series. Plasmid PMBG2 containing the mouse β^{maj}-globin genomic clone was nick-translated with biotin-11-dUTP and the labelled plasmid purified by exclusion chromatography on Sephadex G-50. The hybridization solution consisted of 0.8 μg/ml of labelled DNA, sheared denatured salmon sperm DNA (500 μg/ml), 4 × SSPE and 10% dextran sulphate which was layered onto the slides and hybridized overnight at 60°C in a humidified chamber. Biotin was detected by streptavidin conjugated with horse radish peroxidase (Detek-1-hrp; Enzo Biochem) followed by staining with Enzo dye to detect horse radish peroxidase [6]. Under these conditions the majority of heterozygous transgenic nuclei stained as a single brown dot and control tissue was consistently negative. (Note that on 5 μ sections not all transgenic cells stain positively because the chromosome segment containing the transgenic insert may be outside the section.)

Demonstration of giant granules in beige mouse tubules. Only the proximal tubules can be firmly assigned to the donor in this model. The markers found in the adult beige nephron were not present in embryonic tissue, and pilot experiments showed that they could only be identified in beige mice over two weeks old. The chimeric kidney was fixed in phosphate-buffered 10% formalin overnight at room temperature and embedded in paraffin wax. Five micron sections were mounted on gelatin-coated glass slides, deparaffinized and passed through graded ethanols. Three methods were used for detection as follows.

(a) Autofluorescent granules were viewed on sections mounted in phosphate-buffered saline. An epifluorescence microscope was used to examine sections using ultraviolet excitation (330 to 380 nm excitation filter and 420 nm absorption filter). Under these conditions beige granules autofluoresce bright yellow and appear as large, irregularly distributed granules which are most prominent in the cytoplasm of proximal tubules at the cortico-medullary junction [7] (Fig. 1b). Kidneys of the recipient mice either lacked the marker (Fig. 1c) or rarely

Table 1. Results of chimeric kidneys

Recipient kidney	Donor metanephros	Number surviving >24 hours	Successful engraftment	Evidence of glomerular filtration
Outbred Q strain neonate	Line 83, Transgenic for β-globin × CBA	9	5	Not examined
C57BLGJ neonate	Beige mouse C57BL6 bg^J/bg^J	15	5	2
C57BL6J adult	Beige mouse C57BL6 bg^J/bg^J	8	6	0

showed very fine, diffuse granularity localized near the cell apex.

(b) The large lysosomes were also identified histochemically in the beige tissue by staining for β-galactosidase. After removal of the paraffin and hydration through graded alcohols, sections were immersed for 10 minutes in acetate buffer, pH 5.4 at 4°C, then for a further 10 minutes in similar solution containing 0.01% sodium deoxycholate and 0.02% nonidet P 40 (Sigma Chemical Co., St. Louis, Missouri, USA). The sections were then incubated overnight at 37°C in buffer containing 200 μg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside; Sigma), 0.02 M potassium ferrocyanide and 0.02 M potassium ferricyanide [14]. β-galactosidase activity was identified by a bright indigo color on tissue sections. Kidneys from adult beige mice displayed large intracytoplasmic granules with a similar cellular distribution to the autofluorescent granules. The beige mouse kidney contains twice as much enzyme activity as congenic C57BL/6 mice [8], and under the incubation conditions used, the kidneys of C57BL/6J recipients showed no staining.

(c) Less commonly, the giant granules stain with PAS reagent [10] and show a similar size and intracellular distribution to the autofluorescent granules.

Results

Control operations

Histological evaluation of control-operated kidneys either showed no abnormality or revealed small superficial scars, perpendicular to the renal capsule. These areas contained atrophied glomeruli and tubules. Examination of the scars by epifluorescence microscopy revealed occasional large autofluorescent areas 20 to 50 μ in diameter which appeared in atrophic tubules and presumably represented phagocytosed cell debris.

Chimeric kidneys

General morphology. Ten of the 24 mice which survived the neonatal operation showed evidence of transplanted tissue within the left kidney (Table 1). The embryonic implant developed as a nodule within the cortex and by 16 to 30 days increased in size to a maximum diameter of 1.5 mm and occupied a region of cortex extending from the surface to medulla. The implant was estimated to occupy 1 to 4% of the total kidney volume. This figure may be an underestimate because in the beige tissue implants, only areas containing proximal tubules could be firmly assigned to the donor. The transplanted tissue appeared as a rounded area with glomerular and tubular elements separated by small, basophilic mesenchyme-like cells. In the eight transplants performed in adult animals, the tissue grew as a tumor-like nodule under the capsule of the kidney and showed poor cellular differentiation compared with implants which grew in the neonatal kidneys.

Glomeruli. Sections taken through the widest diameter of the implant generally contained 10 to 30 glomeruli (Fig. 1d). Often, parts of the implant extended to the cortico-medullary junction where collections of donor glomeruli were flanked by host proximal tubules. In some areas within the implant, glomeruli clearly had open capillary loops and contained red blood cells (Fig. 1d, 1e, 1f), whereas in others, smaller, non-vascular glomeruli were seen which appeared as a cluster of epithelial type cells surrounding an area of amorphous eosinophilic material (Fig. 1d).

Tubules. Donor proximal tubules, were usually smaller than the proximal tubules of the surrounding host kidney and possessed PAS-positive brush border membranes. They demonstrated typical β-galactosidase (Fig. 1f, 1g), PAS (Fig. 2a) and autofluorescent (Fig. 2c, 2d, 2e) markers (in beige tissue) or a positive nuclear stain by in situ hybridization (transgenic) (Fig. 2b). Direct continuity between donor glomerulus and proximal tubule was seen only rarely (Fig. 1f).

Many tubules lacked differentiated proximal tubular characteristics. Some were poorly formed and had no patent lumen (Fig. 2b), and others appeared to be of "distal" type with flattened epithelia and open lumens without a brush border. In two chimeric kidneys linear structures of donor origin resembling loops of Henle were seen extending from the region of the implant into the medullary regions (Fig. 2f and 2g).

Glomerular function. This was evaluated on five beige tissue transplants by FITC-dextran injection. In two chimeric kidneys clear evidence of filtration of fluorescein into the lumen of beige tubules was seen, indicating that these tubules were connected to a filtering glomerulus (Fig. 2c, 2d, 2e). In these two kidneys, the numbers and mature morphology of donor proximal tubules were especially marked.

Discussion

The present study demonstrates the feasibility of creating a chimeric kidney, that is, an organ constituted by cells derived from more than one fertilized ovum, by implantation of embryonic metanephros into the renal cortex of neonatal animals. These transplants grow in size and contain differentiated nephrons. Even though we did not perform an ultrastructural analysis, the presence of mature features in donor proximal tubules by light microscopy (such as, brush border membrane and adult-type beige markers) indicates that differentiation occurred within the host since such features were absent in the metanephros of 13 to 16 day embryos. In contrast, in studies which have either transplanted rodent metanephros onto the avian chorioallantoic membrane [15], or induced embryonic mesenchymal growth in vitro [16], tubule formation and differentiation has been poor by comparison with our model, although highly advanced proximal tubular differentiation has been demonstrated in metanephric organ culture [11].

The profusion of mature glomeruli within some implants also indicates that they, too, have formed after transplantation since the implanted tissue rarely contained more than a few mature-type glomeruli located principally in the cortico-medullary

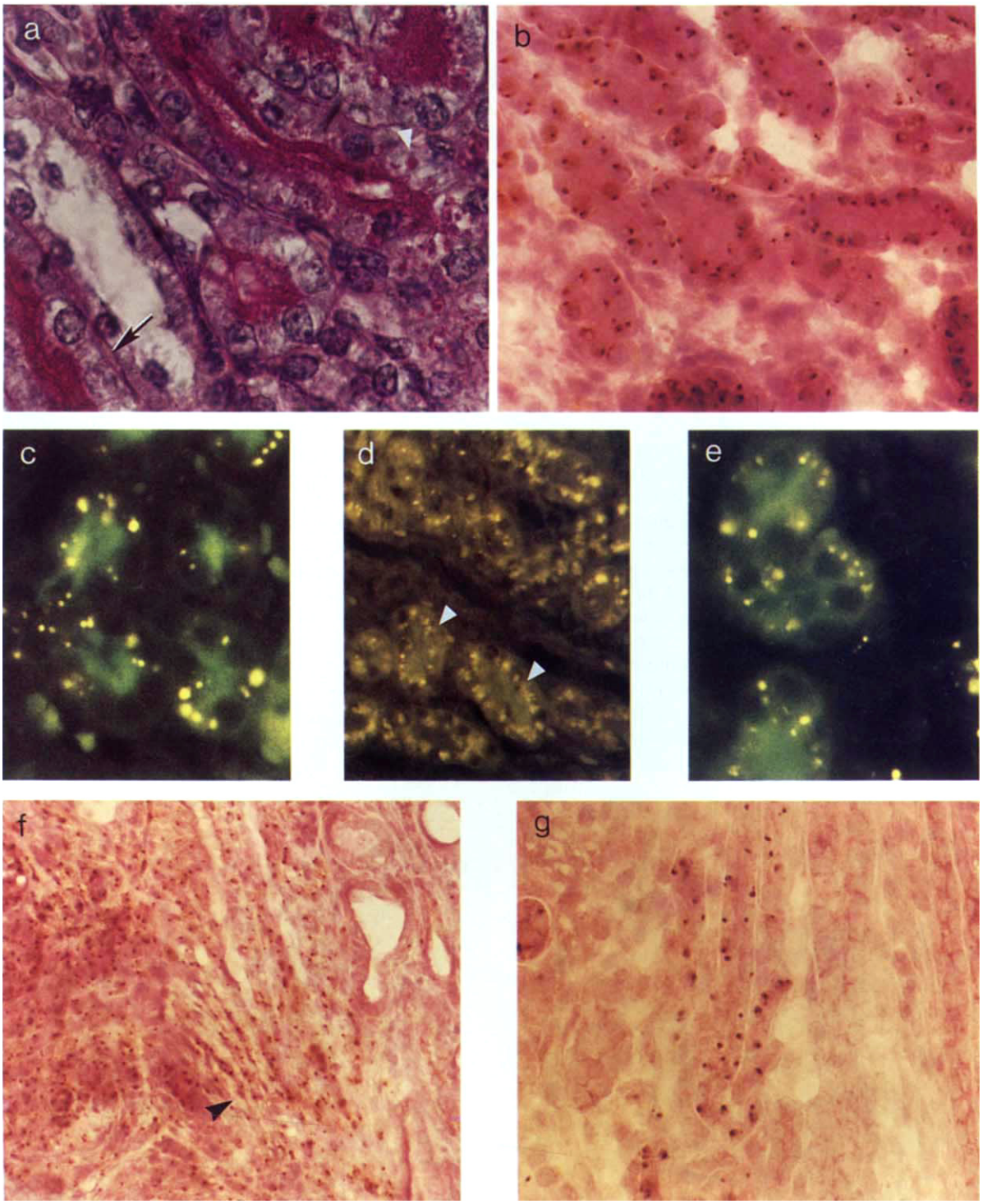

Fig. 2. a. *Chimeric kidney*. On right of panel is a donor beige-type proximal tubule with PAS-positive granules in cytoplasm (white arrow). At lower left is a recipient tubule which lacks these granules (black arrow). (PAS × 800) **b.** Chimeric kidney. Donor tubules of transgenic origin without patent lumen. (× 320) **c.** Chimeric kidney. Beige donor proximal tubules contain both autofluorescent yellow granules within cytoplasm and FITC-dextran green fluorescein within their lumens, indicating that tubules are patent and connected to a filtering glomerulus. (× 800) **d.** Chimeric kidney. Lower power view of mass of beige donor tubules. Only those in lower part of panel are filtering and have fluorescence within lumens (arrows). (× 320) **e.** Chimeric kidney. As for (c) showing a different filtering nephron. (× 800) **f.** Chimeric kidney. Left part shows donor transgenic tissue with long tubular structures (arrow). Recipient tissues is seen on right. (× 128) **g.** Chimeric kidney. Tubules derived from transgenic metanephros are shown extending downwards into the medulla of the recipient. (× 320)

region of the metanephros [16]. The presence of vascular loops containing erythrocytes in donor glomeruli indicated continuity with the vasculature of the host kidney. Blood vessels do not develop in glomeruli when the metanephros grows in organ culture [11], but, when this structure is grafted onto avian chorioallantoic membrane, glomeruli derive their capillary tufts by ingrowth from the host [15]. Similar experiments using the more mature metanephros (in which some glomeruli have become vascularized), produce glomeruli with double tufts which may represent capillaries derived from both the host and the donor tissue [15]. The present experiment cannot establish the origin of the vascular supply to glomeruli in the chimeric kidney with certainty since the resolution of the in situ hybridization is not adequate to pinpoint a signal over an endothelial cell. However, if this problem of cellular definition could be overcome, the current model would provide a suitable in vivo system with which to study glomerular angiogenesis.

The appearance of capillaries within transplanted glomeruli was obviously not adequate, per se, to establish that ultrafiltration occurs, especially since angiogenic factors (if they are present) tend to attract capillary endothelial cells of venular origin [17, 18] in which cases the appropriate forces for ultrafiltration would be absent. With this problem in mind, we used the FITC-dextran method to demonstrate ultrafiltration by donor nephrons. In two chimeric kidneys we found fluorescein within the lumens of donor-type proximal tubular cells, showing that some of the donor glomeruli can, indeed, filter plasma. An alternative explanation is that the donor proximal tubules established continuity with filtering host glomeruli, a highly unlikely possibility. Theoretically, it might be possible for transplanted glomeruli to filter at low rates into blind-ended tubules which reabsorb all the filtrate. Although the current study cannot provide evidence of appropriate connections between donor nephrons and recipient collecting tubes, this might be demonstrated by isolated nephron microdissection. Finally, the non-perfusion fixation protocols used in the study might have affected the number of "open" tubular lumens in the transplanted tissue.

When the adult is used as host for the metanephros, the tissue is not retained within the kidney but is extruded to grow as a mass under the renal capsule resembling a poorly-differentiated tumor. Thus the neonatal kidney, which has a rim of undifferentiated cortex, can facilitate the differentiation of an embryonic implant, but this ability is lacking in the fully-differentiated adult kidney.

It should be emphasized that the experiments which are described herein were only sporadically successful as shown in Table 1. Many operated animals show no evidence of an implant when sacrificed at two to four weeks. We speculate that these failures may be caused by the fragile implants being forced out of the host kidney upon its restoration to the abdominal cavity. Secondly, not all glomeruli become vascularized and, if the production of an angiogenic factor by the glomerular capsule is required for vascularization, this does not occur uniformly. Similar considerations apply to the formation of tubules which did not attain the typical adult morphology in every chimeric kidney. The relative rarity of glomerular filtration by donor nephrons is not surprising, considering that a connection with the host's high-pressure arteriolar system and fusion with the host's collecting system are both probably essential to permit filtration and flow of the tubular fluid. There appears to be no correlation of the successful engraftment of a transplant with the age of donor tissue in our current study. Although fetal pancreatic islets [19] and neurons [20] have been transplanted into adults to form functioning tissue, the current experiments probably represent the most complex embryonic tissue that has formed a functioning organ after implantation in vivo.

The success of these initial experiments raises intriguing possibilities. First, it may become possible to increase the number of functioning nephrons of donor origin in a chimeric kidney by pre-incubation of the metanephros with specific growth factors such as EGF, TGFβ and retinoic acid [21, 22], and we have successfully maintained metanephric tissue in organ cultures for 24 hours and have found that it retains its viability when transplanted into a host kidney. (A.S. Woolf and L.G. Fine, unpublished observations). Second, the ability to manipulate the tissue prior to implantation raises the possibility of using gene transfer techniques on mammalian kidney [23]. Since the donor tissue is at a stage of rapid cell proliferation, successful gene transfer would be multiplied many times in the fully developed tissue. If donor nephrons containing a new or an altered gene product are able to target such molecules to the appropriate membrane for export, it becomes feasible to consider the possibility of introducing a paracrine system into the kidney or a means for delivering specialized molecules into the urine.

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